

# Influence of the Diversity of Bacterial Isolates from Drinking Water on Resistance of Biofilms to Disinfection<sup>▽</sup>

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**Single- and multispecies biofilms formed by six drinking water-isolated bacterial species were used to assess their susceptibilities to sodium hypochlorite (SHC). In general, multispecies biofilms were more resistant to inactivation and removal than single biofilms. Total biofilm inactivation was achieved only for *Acinetobacter calcoaceticus* single-species biofilms and for those multispecies biofilms without *A. calcoaceticus*. Biofilms with all bacteria had the highest resistance to SHC, while those without *A. calcoaceticus* were the most susceptible. *A. calcoaceticus* formed single biofilms susceptible to SHC; however, its presence in multispecies biofilms increased their resistance to disinfection.**

The control of drinking water (DW) quality in distribution systems is a major technological challenge to the water industry. DW networks can be regarded as biological reactors which host a wide variety of microorganisms (bacteria, protozoa, and fungi), both in the bulk water and on the pipe surfaces. In DW distribution systems (DWDS), *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*/*Corynebacterium*, *Bacillus*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Methylobacterium*, *Moraxella*, *Pseudomonas*, *Serratia*, *Staphylococcus*, *Mycobacterium*, *Sphingomonas*, and *Xanthomonas* have been the predominant bacterial genera detected (2, 3). The Gram-negative bacteria are predominant over the Gram-positive bacteria, and *Pseudomonas* is the most abundant bacterial organism in supply systems, regardless of the water source. Most of the biomass present in these DWDS is located at the pipe walls. Flemming et al. (7) proposed that 95% of the bacteria were adhered to the surface of pipelines and only 5% were present in the bulk water. The presence and significance of biofilms in DWDS have been repeatedly reported (16, 18). Biofilm growth and detachment contribute to the increase in the number of cells in bulk water (5). Some of those microorganisms can be pathogens. Commonly encountered waterborne pathogens are *Burkholderia pseudomallei*, *Campylobacter* spp., *Escherichia coli*, *Helicobacter pylori*, *Legionella pneumophila*, *Mycobacterium avium*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, and *Vibrio cholerae* (32). Therefore, biofilm control is important for technical, esthetic, regulatory, and public health reasons.

Chlorine disinfection is a key step in the biofilm control process. Residual concentrations must be kept below guidelines to lower the potential to form harmful disinfection by-products (20). Chlorine, a strong oxidizing agent, is the most commonly used disinfectant due to its effectiveness, stability,

easy of use, and low cost. However, biofilm formation and resistance to disinfection have been recognized as important factors that contribute to the survival and persistence of microbial contamination in DW (2). Research into DW biofilm control will help to determine optimal disinfection parameters and lead to knowledgeable decisions regarding the management of DW distribution networks that will guarantee microbe-safe and high-quality DW. The main purpose of this work was to understand the impact of the microbial diversity of DW biofilms on their resistance to disinfection. The effects of sodium hypochlorite (SHC) on the control of single- and multispecies biofilms formed by DW-isolated bacteria, recognized as problematic opportunistic bacteria and with the potential to cause public health problems, were studied.

The bacteria used throughout this work were isolated from a model laboratory DWDS and identified as described previously by Simões et al. (23). The assays were performed with 6 representative DW-isolated bacteria, *Acinetobacter calcoaceticus*, *Burkholderia cepacia*, *Methylobacterium* sp., *Mycobacterium mucogenicum*, *Sphingomonas capsulata*, and *Staphylococcus* sp. Bacteria were grown overnight in batch cultures using 100 ml of R2A broth at room temperature ( $23 \pm 2^\circ\text{C}$ ) and under agitation (150 rpm). Afterwards, the bacteria were harvested by centrifugation (20 min at  $13,000 \times g$ ,  $4^\circ\text{C}$ ), washed three times in 0.1 M saline phosphate buffer, and resuspended in a certain volume of R2A broth to obtain a cellular density of  $1 \times 10^8$  cells/ml. Biofilms were developed according to the modified microtiter plate test proposed by Stepanović et al. (28) using R2A broth as growth medium. Single-species biofilm formation was carried out with the six DW-isolated bacteria, and multispecies biofilms were developed at seven different bacterial combinations: one mixture of all six bacteria and six combinations with a mixture of five distinct bacteria through a strain exclusion process (biofilm formation in the absence of a specific strain, obtaining distinct species combinations) (25). For each condition, the wells of sterile 96-well flat-tissue culture plates (polystyrene; Orange Scientific) were filled under aseptic conditions with 200  $\mu\text{l}$  of a cell suspension ( $10^8$  cells/ml). Multispecies biofilms were developed with equal

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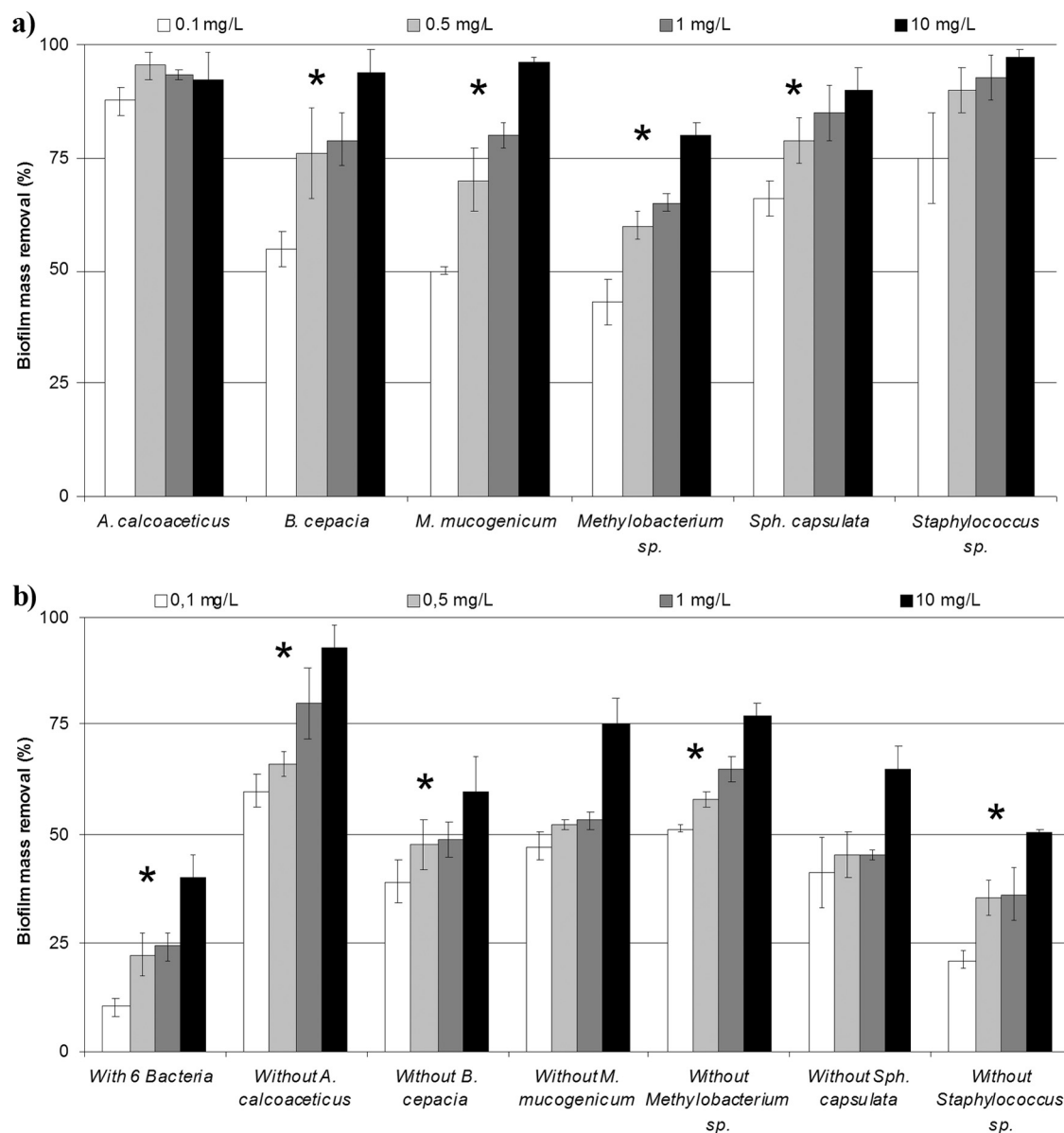


FIG. 1. Percentage of biofilm mass removal for single-species (a) or multispecies (b) biofilms after their exposure to several SHC concentrations. Means  $\pm$  SDs for at least three replicates are illustrated. “\*” indicates significant influence ( $P < 0.05$ ) of SHC concentrations in biofilm removal.

initial cell densities of each isolate. Negative controls were obtained by incubating the wells with R2A broth without adding any bacterial cells. To promote biofilm formation, plates were incubated aerobically on an orbital shaker at 150 rpm and at room temperature for 72 h. The growth medium was carefully discarded and freshly added every 24 h. All experiments were performed in triplicate with at least three repeats. After the biofilm formation period, the content of each well was removed and the wells were washed three times with 250  $\mu$ l of sterile distilled water to remove reversibly adherent bacteria. The remaining attached bacteria on the inner walls of the wells were submitted to the disinfection assay. A stock solution of SHC was prepared by diluting a commercially available solution (Sigma, Portugal) with sterile distilled water. Disinfectant solutions at various concentrations (0.1, 0.5, 1, and 10 mg/liter)

were prepared on the day of use and stored in the dark at 4°C. The biofilms, immediately after rinsing, were exposed to several independent SHC concentrations. At least 16 wells of 96-well microtiter plate were filled under aseptic conditions with 250  $\mu$ l of each concentration of SHC. In addition to the treated wells, control (untreated) biofilm wells were also used for each biofilm condition. The SHC solutions remained in contact with the biofilms for 1 h but were removed and refreshed every 20 min during the 1-h treatment period. SHC solutions were refreshed due to the high density of cells in the biofilms and the low volumes applied for treatment (21). In order to improve the contact of biofilm cells with SHC, the microtiter plates were incubated on a shaker at 150 rpm and at room temperature. After treatment, the disinfectant solutions were removed by rinsing the wells twice with 250  $\mu$ l of sodium

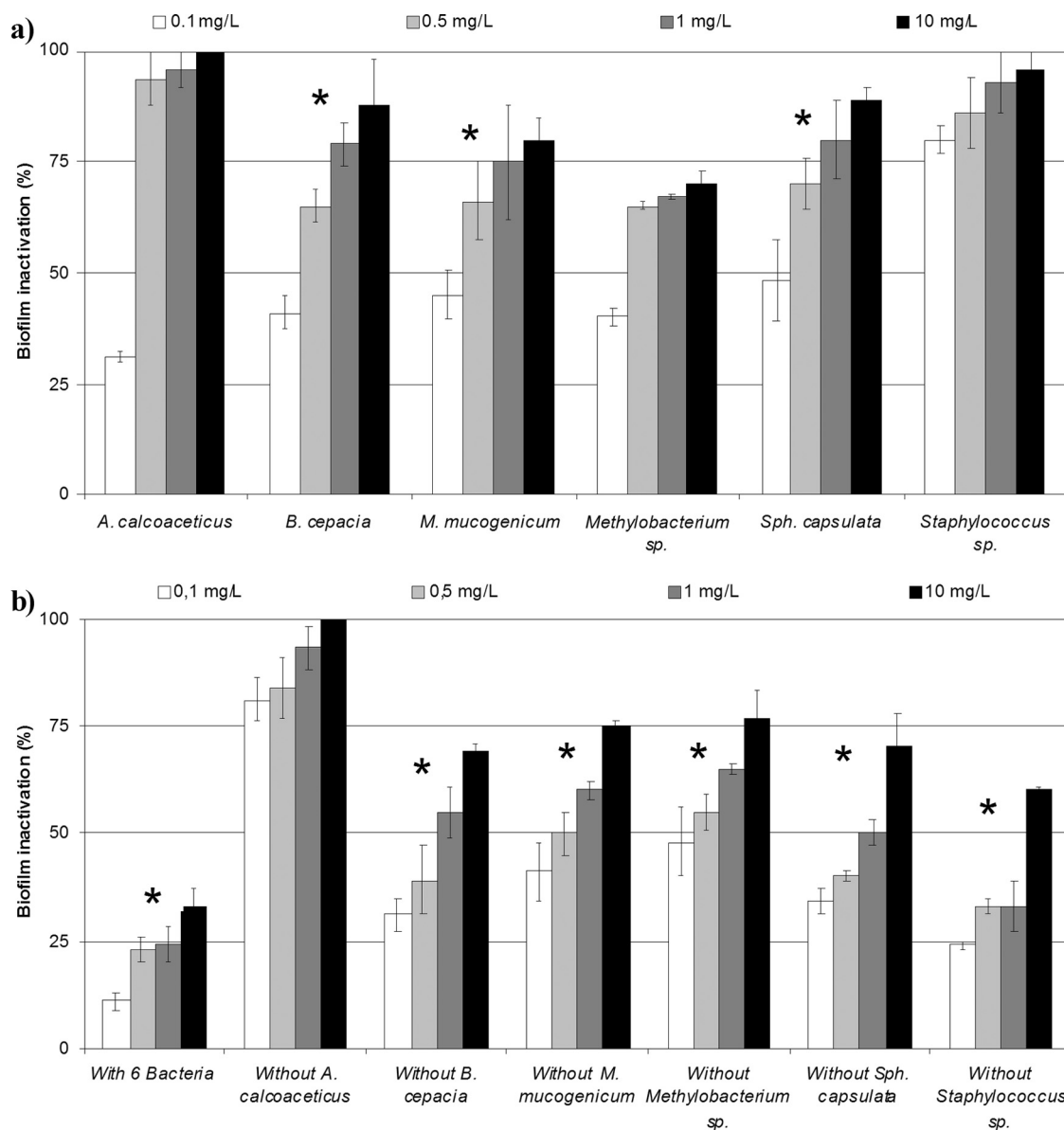


FIG. 2. Percentage of biofilm inactivation for single-species (a) or multispecies (b) biofilms after their exposure to several SHC concentrations. The means  $\pm$  SDs for at least three replicates are illustrated. “\*” indicates significant influence ( $P < 0.05$ ) of SHC concentrations in biofilm inactivation.

thiosulfate solution (Merck, VWR, Portugal) at 0.5% (wt/vol) in sterile distilled water to quench the activity of the disinfectant and one time with 250  $\mu$ l of sterile distilled water. Afterwards, the biofilms were analyzed in terms of biomass, metabolic activity, cultivability, and viability. Biofilm mass was assessed by crystal violet staining (24), metabolic activity was determined by the 3,3'-[1[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) colorimetric method (24), cultivability was assessed in R2A (22), and viability was assessed using the L-7012 Live/Dead (L/D) *BacLight* bacterial viability kit (27).

The SHC effectiveness (removal and inactivation) was assessed based on the absorbance values of the blank, the control experiment, and the treated biofilm: biofilm removal/inactivation (%) =  $\{[(C - B) - (T - B)] / (C - B)\} \times 100$ . *B* indicates

the average absorbance for the blank wells (without bacteria), *C* indicates the average absorbance for the control wells (untreated biofilms), and *T* indicates the average absorbance for the SHC-treated wells (19).

Biofilm control in terms of cultivability (CFU) and viability (L/D) was calculated by the following expression: biofilm cultivability/viability reduction (%) =  $\{[CFUs \text{ or } L/D_{\text{control}} - CFUs \text{ or } L/D_{\text{disinfection}}] / CFUs \text{ or } L/D_{\text{control}}\} \times 100$ .

The data were analyzed by the nonparametric Wilcoxon test based on a confidence level of  $\geq 95\%$ .

The present study has implications for understanding the role of microbial diversity on biofilms formed by DW-isolated bacteria in their susceptibility to SHC. The SHC concentrations used were those usually present in DWDS, with the exception of the highest concentration (10 mg/liter). This was

used to promote significant biofilm removal and inactivation results, taking into account the high cell densities of the biofilms formed on the microtiter plates (increasing the ratio of SHC per amount of biofilm). According to the World Health Organization (32), 2 to 3 mg/liter of chlorine should be added to water in order to provide satisfactory disinfection and a residual concentration along DWDS. However, the maximum amount of chlorine one can use is 5 mg/liter (32). This study was developed using polystyrene microtiter plates, which are the most frequently used bioreactor system for studying biofilm formation and disinfection, providing reliable comparative data (19, 21). Microtiter plates can be used as a rapid and simple method to screen the differences in efficiency of chlorine to remove and kill different biofilms. Polystyrene has physicochemical surface properties similar to those of other materials used in DWDS, such as stainless steel and polyvinylchloride (23).

The biofilm removal results demonstrate that *Methylobacterium* sp. formed the most resistant biofilms (Fig. 1a). *A. calcoaceticus* formed the biofilms most susceptible to SHC up to a 1-mg/liter concentration, and *Staphylococcus* sp. biofilms were the most susceptible at the highest concentration. For multispecies biofilms (Fig. 1b), the order of susceptibility (from less to more susceptible) for all the SHC concentrations was the following: the biofilm with 6 bacteria, that without *Staphylococcus* sp., that without *B. cepacia* or *S. capsulata*, that without *M. mucogenicum*, that without *Methylobacterium* sp., and that without *A. calcoaceticus*. In comparing single- and multispecies biofilms (Fig. 1a and b), almost all multispecies biofilms were more resistant to removal than the single biofilms ( $P < 0.05$ ), except those multispecies biofilms without *M. mucogenicum* and without *Methylobacterium* sp. with 0.1 mg/liter of SHC and multispecies biofilms without *A. calcoaceticus* for all the SHC concentrations tested ( $P > 0.05$ ). These biofilms were more susceptible to chlorine than some of the single biofilms (*Methylobacterium* sp. [all concentrations], *M. mucogenicum* [0.1 mg/liter], *B. cepacia* [0.1 and 1 mg/liter], and *S. capsulata* and *A. calcoaceticus* [10 mg/liter]).

Biofilm inactivation increased with the SHC concentration for all the biofilms. *A. calcoaceticus* single biofilms presented the highest inactivation values for all the concentrations tested, with the exception of 0.1 mg/liter (Fig. 2a). For this concentration, *A. calcoaceticus* formed biofilms with the highest resistance to inactivation, while *Staphylococcus* sp. biofilms were the most susceptible. *Methylobacterium* sp. biofilms were the most resistant to disinfection at SHC concentrations higher than 0.1 mg/liter. The sequence of resistance to inactivation for SHC concentrations of  $\geq 1$  mg/liter was the following: *Methylobacterium* sp. was more resistant than *M. mucogenicum*, which was more resistant than *B. cepacia*, followed by *S. capsulata*, followed by *Staphylococcus* sp., followed by *A. calcoaceticus*. *A. calcoaceticus* biofilms reached total inactivation with SHC at 10 mg/liter. For multispecies biofilms (Fig. 2b), the bacterial combination with the six bacteria was the most resistant to inactivation, followed by multispecies biofilms without *Staphylococcus* sp. The least resistant were the multispecies biofilms without *A. calcoaceticus*, followed by the biofilms without *Methylobacterium* sp., for all SHC concentrations. The multispecies biofilms with all six bacteria had the highest resistance to disinfection (even for high SHC concentrations, only a 60% biofilm inactivation was obtained). Those without

TABLE 1. Initial (before disinfection) counts of single- and multispecies cultivable and viable biofilm cells<sup>a</sup>

| Biofilm description                 | Cultivable cell count | Viable cell count |
|-------------------------------------|-----------------------|-------------------|
| Single biofilms                     |                       |                   |
| <i>A. calcoaceticus</i>             | 5.08 $\pm$ 0.43       | 6.33 $\pm$ 0.05   |
| <i>B. cepacia</i>                   | 5.24 $\pm$ 0.33       | 6.41 $\pm$ 0.44   |
| <i>M. mucogenicum</i>               | 4.37 $\pm$ 0.70       | 6.06 $\pm$ 0.66   |
| <i>Methylobacterium</i> sp.         | 6.58 $\pm$ 0.55       | 7.69 $\pm$ 0.14   |
| <i>S. capsulata</i>                 | 5.70 $\pm$ 0.16       | 6.88 $\pm$ 0.32   |
| <i>Staphylococcus</i> sp.           | 6.00 $\pm$ 0.38       | 7.21 $\pm$ 0.46   |
| Multispecies biofilms               |                       |                   |
| With 6 bacteria                     | 6.87 $\pm$ 0.23       | 7.91 $\pm$ 0.31   |
| Without <i>A. calcoaceticus</i>     | 6.88 $\pm$ 0.11       | 7.97 $\pm$ 0.40   |
| Without <i>B. cepacia</i>           | 7.03 $\pm$ 0.28       | 8.11 $\pm$ 0.26   |
| Without <i>M. mucogenicum</i>       | 7.15 $\pm$ 0.36       | 8.51 $\pm$ 0.08   |
| Without <i>Methylobacterium</i> sp. | 6.70 $\pm$ 0.45       | 7.89 $\pm$ 0.68   |
| Without <i>S. capsulata</i>         | 6.70 $\pm$ 0.16       | 7.78 $\pm$ 0.41   |
| Without <i>Staphylococcus</i> sp.   | 7.23 $\pm$ 0.41       | 8.31 $\pm$ 0.55   |

<sup>a</sup> Values are expressed as log CFU/cm<sup>2</sup> or log viable cells/cm<sup>2</sup>  $\pm$  SD.

*A. calcoaceticus* had a high susceptibility to SHC even for small concentrations (biofilm inactivation was always higher than 80%; total biofilm inactivation for SHC occurred at 10 mg/liter). In general, the multispecies biofilms were more resistant to inactivation than the single ones (Fig. 2a and b). Multispecies biofilms without *A. calcoaceticus* were the most relevant exception. Those biofilms were more susceptible to disinfection at some SHC (0.1 mg/liter) concentrations than the single-species biofilms ( $P < 0.05$ ).

The single- and multispecies biofilms were also characterized in terms of cultivable and viable cells (Table 1). The number of viable cells was higher than the number of cultivable cells for all single- and multispecies biofilms (magnitude of difference of 1 to 2 logs of cells/cm<sup>2</sup>). The multispecies biofilms always displayed higher numbers of cultivable and viable cells than the single species. Also, L/D results demonstrated that before disinfection, almost all the bacteria in the several single- and multispecies biofilms were in a viable state (99.9%  $\pm$  0.003%). The differences provided by viability and cultivability methods allowed the assessment of the number of noncultivable but metabolically active cells, classically called "viable but noncultivable" (VBNC), which exist in response to chlorine stress (10). These cells can be either temporarily noncultivable, cultivable under other culture conditions, or simply dead cells (15). Lindsay et al. (13) highlighted the importance of taking into account the injured cell population during disinfection since such populations may recover and recolonize the surfaces. Plate count techniques are known to be inefficient in the detection of disinfectant-injured bacteria and can overestimate disinfection (26). However, even if the cell counts based on culture methods underestimate the number of bacteria, some authors argue that they could be used as a general indicator that demonstrates the efficiency of disinfection in DWDS (1).

Biofilm cultivability and viability after disinfection provided results comparable with those obtained by XTT staining for all the biofilms ( $P > 0.05$ ). It was also verified that biofilm cultivability and viability decreased with the increasing SHC concentration. Comparing the values obtained for metabolic inactivation (Fig. 2), cultivability reduction (Fig. 3), and viability

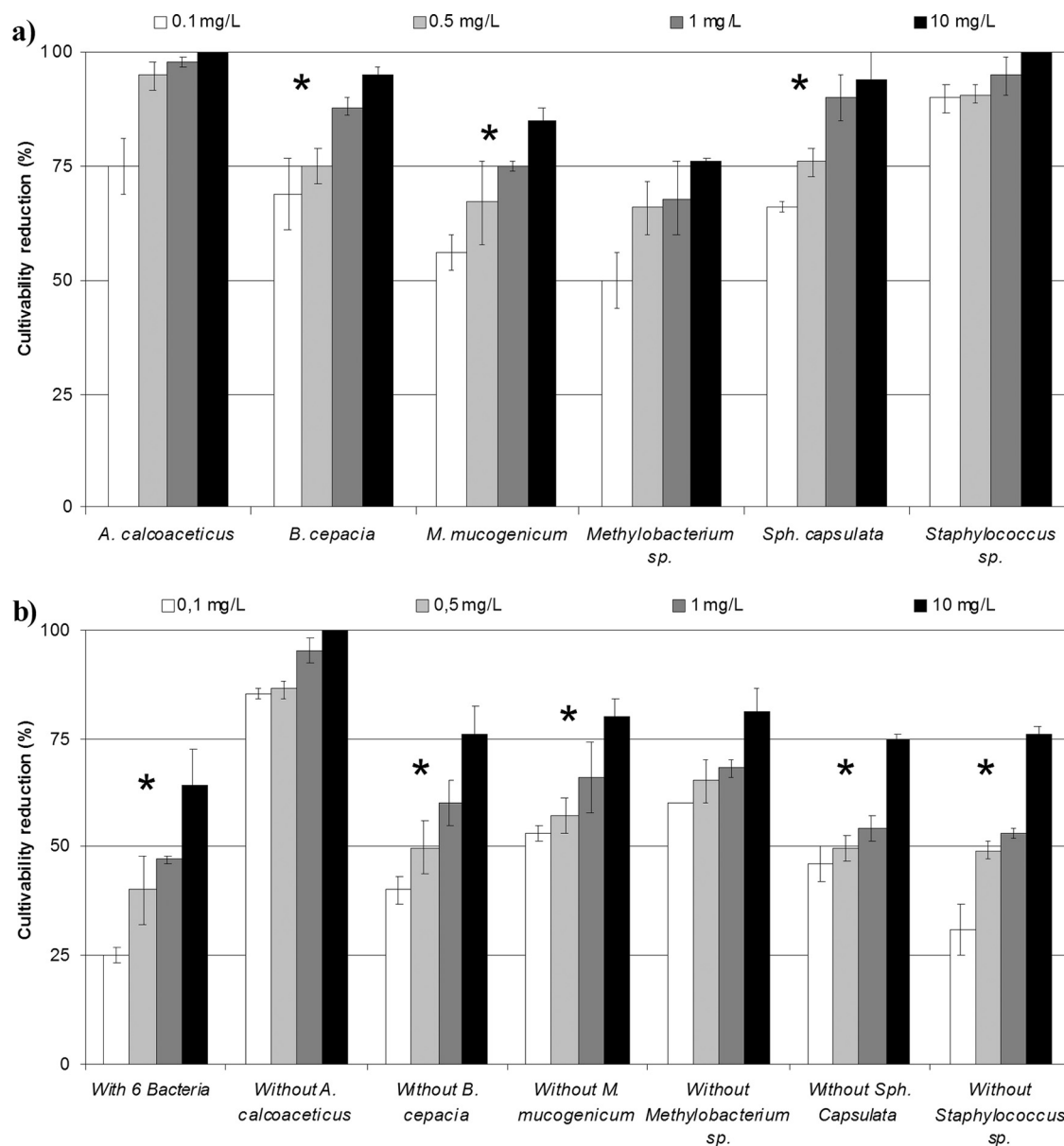


FIG. 3. Percentage of cultivability reduction for single (a) or multispecies (b) biofilms after their exposure to several SHC concentrations. The means  $\pm$  SDs for at least three replicates are illustrated. “\*” indicates significant influence ( $P < 0.05$ ) of SHC concentrations in biofilm cultivability reduction.

reduction (Fig. 4), the cultivability results provided the most promising biofilm control results for all the scenarios tested. This is apparently related to the existence of VBNC cells. According to Thomas et al. (30), these cells constitute the most numerically significant and persistent subpopulation within the aquatic systems.

The increased resistance of multispecies biofilms can be partly explained by the higher cell densities relative to those of single biofilms. The cell densities of multispecies biofilms were higher than those of the single ones for all the biofilms tested. Other potential reasons for the increased resistance of biofilm cells to antimicrobials include the difficulty in penetration of the matrix surrounding the biofilms by a disinfectant, the altered microenvironment, which in turn contributes to slow microbial

growth, the acquisition of resistance phenotypes, and the existence of persistent cells (6, 12, 21). Also, the interactions in multispecies biofilms may influence each other not only with respect to attachment capabilities but also in susceptibility or resistance to a disinfectant (4, 13, 27). According to Shakeri et al. (21), the higher resistance of multispecies biofilms than of single-species biofilms depends on the variation in the species incorporated and the role of each species. This may be due to the resistance of only one or two key strains. Leriche and Carpentier (10) demonstrated that *Pseudomonas fluorescens* and *Salmonella enterica* serovar Typhimurium in biofilm enhanced each other's survival following chlorine treatment. The coculturing of the two bacteria in biofilm enhanced resistance of the individual strains to disinfection. *Staphylococcus sciuri*



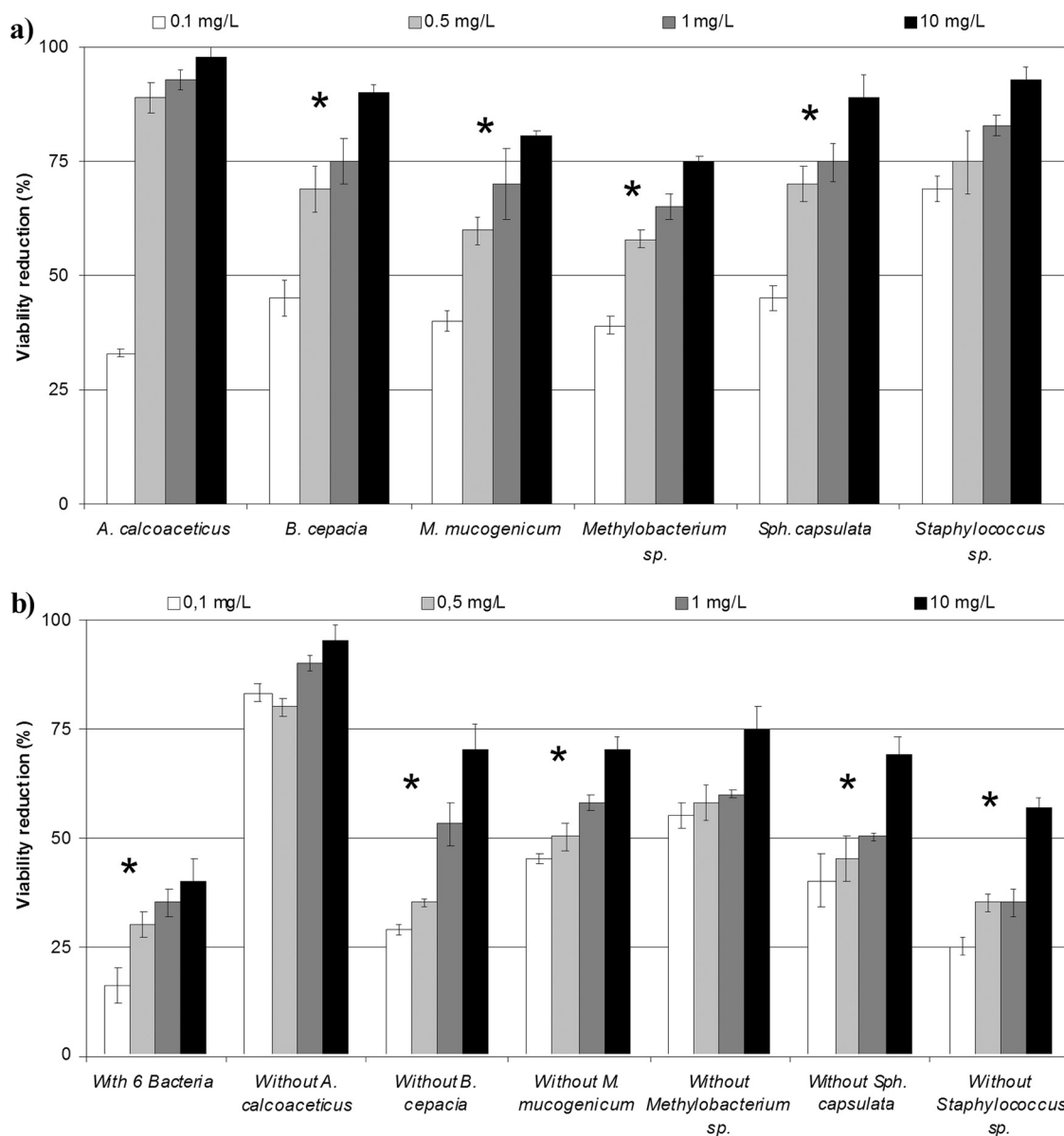


FIG. 4. Percentage of viability reduction for single-species (a) or multispecies (b) biofilms after their exposure to several SHC concentrations. The means  $\pm$  SDs for at least three replicates are illustrated. “\*” indicates significant influence ( $P < 0.05$ ) of SHC concentrations in reduction of biofilm viability.

was also found to protect *Kocuria* species microcolonies against a chlorinated alkaline solution (11). Other apparent protective effects caused by bacterial association have been mentioned (13, 27). The synergistic species association found in this study, in addition to other well-described biofilm-specific antimicrobial resistance mechanisms (6, 14), could at least partly explain the survival of complex multispecies biofilms in adverse environments.

The comparison of the SHC susceptibilities of multispecies biofilms shows that biofilms composed by the six different species had the highest resistance to removal and inactivation. In fact, the results demonstrate that biofilm species association/diversity promotes community stability and functional resilience even after SHC treatment. Biofilms in the absence of

*Staphylococcus sp.* had a significant resistance to SHC. On the other hand, *Staphylococcus sp.* single biofilms were highly susceptible to SHC. This result is arguably related to the higher susceptibility of Gram-positive bacteria to multitarget antimicrobials comparatively to that of Gram-negative bacteria (31). Whereas the envelopes of Gram-positive bacteria consist of the cytoplasmic membrane surrounded by a thick peptidoglycan wall, the envelopes of Gram-negative bacteria possess an external layer, the outer membrane, which provides an extra barrier against antimicrobials. The most susceptible multispecies biofilms were those lacking *A. calcoaceticus*, *Methylobacterium sp.*, and *M. mucogenicum*. The absence of these bacteria in the multispecies biofilm increased the susceptibility to SHC. *A. calcoaceticus* biofilms were significantly affected by chlorine

even at small concentrations. This bacterium was one of the most susceptible. On the other hand, multispecies biofilms that lacked *A. calcoaceticus* led the most SHC-susceptible biofilms and showed a decreased ability to recover from disinfection (results not shown). This can be explained by the role of *A. calcoaceticus* as a bridging bacterium in this microbial community. In a previous study, it was demonstrated that this bacterium has the ability to coaggregate with almost all other bacteria (except *Methylobacterium* sp.), and its presence in a multispecies community represented a colonization advantage (25). This bacterium may facilitate the association of other species that do not coaggregate directly with each other, increasing the opportunity for metabolic cooperation. Bacterial coaggregation in well-established microbial biofilm communities seems to be one potential synergistic interaction that not only promotes their growth but also improves their resistance to SHC disinfection (17). *Methylobacterium* sp. and *M. mucogenicum* single biofilms were the most resistant to SHC. The increased resistance demonstrated by these bacteria can arguably be related to their ability to form biofilms with the highest cell densities. Also, *Methylobacterium* sp. had the lowest doubling time (results not shown). According to Taylor et al. (29), the more slowly growing strains are more resistant to chlorine than the rapidly growing strains. Hirashi et al. (8) verified that *Methylobacterium* isolates derived from chlorinated water supplies exhibited higher resistance to chlorine than other isolates from different environments. *Mycobacteria* are among the least susceptible cell types due to the innate presence of a waxy cell envelope (9).

In conclusion, knowledge of biofilm microbial diversity and behavior can contribute to the design of effective control strategies (able to control the key microorganisms in the resistance and resilience of a biofilm, such as *A. calcoaceticus*) that will guarantee safe and high-quality DW. Often the mechanisms responsible for the survival of bacteria in DW supplies are unknown or poorly understood. Some authors already have proposed that this increased resistance to disinfection may result from the microbial diversity and microbial interactions in well-established consortiums adhered on the walls of water pipes (2). To our knowledge, this is the first report providing experimental evidence of the role of the microbial diversity of DW-isolated bacteria biofilms in their resistance to SHC disinfection.

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